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Howard Milne Chandler

**AUSTRALIA**

**Patents Act 1990**

**PROVISIONAL SPECIFICATION**

for the invention entitled:

"A method for detecting blood"

The invention is described in the following statement:

screening for colorectal cancer. These tests typically detect the globin protein of haemoglobin, a protein that does not survive passage through the upper gastrointestinal tract. A positive immunological test therefore indicates lower gastrointestinal bleeding. In common with all immunologically based tests, however, these tests are subject to a "prozone" or "high dose hook" effect, where at high levels of analyte, the test may be inhibited to the extent that heavy bleeding may be missed.

Accordingly, there is a need to develop improved methods of detecting blood in biological samples which methods minimise the incidence of false negative results obtained due to the effects of the prozone phenomenon. In work leading up to the present invention, the inventor has developed a method of screening biological samples for the presence of blood utilising a two part testing procedure which comprises an immunological screen for the presence of the globin component of haemoglobin performed and a non-immunological screen for the haem component of haemoglobin. Accordingly, even if the immunological detection method utilised to screen for globin produces a false negative result due to the presence of high concentrations of globin, the haem test which is not sensitive to the effects of the prozone phenomenon will nevertheless produce a positive result.

## SUMMARY OF THE INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Accordingly, the present invention provides a method of detecting the presence of blood in a biological sample, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix which test matrix comprises first, second and third regions;

(i) applying a gastrointestinal sample to a first region of a test matrix which test matrix comprises first, second and third regions;

(ii) permitting flowing of said gastrointestinal sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin antibody for a time and under conditions sufficient for a globin antiglobin complex to form and detecting said globin antiglobin complex; and

(iii) permitting flowing of said gastrointestinal sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem.

Accordingly, another aspect of the present invention is directed to a method of detecting lower gastrointestinal bleeding, said method comprising the steps of:

(i) applying a faecal sample to a first region of a test matrix which test matrix comprises first, second and third regions;

(ii) permitting flowing of said faecal sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detecting said globin-antiglobin complex; and

(iii) permitting flowing of said faecal sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem;

wherein a positive haem result and a positive globin result is indicative of lower gastrointestinal tract bleeding.

(iii) permitting flowing of said biological sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem.

Preferably, the present invention is directed to a method of diagnosing colorectal cancer, said method comprising the steps of:

(i) apply a faecal sample to a first region of a test matrix which test matrix comprises first, second and third regions;

(ii) permitting flowing of said faecal sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detecting said globin-antiglobin complex; and

(iii) permitting flowing of said faecal sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem;

wherein a positive haem result and a positive globin result is indicative of colorectal cancer.

Accordingly, the present invention more particularly provides a method of detecting the presence of blood in a biological sample, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix which test matrix comprises first, second and third regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detecting said globin-antiglobin complex; and

(iii) permitting flowing of said biological sample to a third region of said test matrix wherein said sample is placed in contact with guaiac or functional equivalent thereof for a time and under conditions sufficient for said guaiac to detect haem.

Reference to "biological sample" should be understood as a reference to any sample of biological material derived from an animal such, but not limited to, mucus, faeces, urine, biopsy specimens and fluid which has been introduced into the body of an animal and subsequently removed such as, for example, the saline solution extracted from the lung following lung lavage or the solution retrieved from an enema wash. The biological sample which is tested according to the method of the present invention may be tested directly or may require some form of treatment prior to testing. For example, a biopsy sample may require homogenisation prior to testing. Further, to the extent that the biological sample is not in liquid form, (for example it may be a solid, semi-solid or a dehydrated liquid sample) it may require the addition of a reagent, such as a buffer, to mobilise the sample. The mobilising reagent may be mixed with the biological sample prior to application of the sample to the test matrix or the reagent may be applied to the sample after the sample has been applied to the test matrix. The use of a mobilising reagent is required to facilitate flowing (wicking) of the sample along the test matrix. Preferably, the biological sample is a gastrointestinal sample. By "gastrointestinal sample" is meant any sample which is derived from the gastrointestinal

and a second region which comprises two sections. The first section of the second region is an area of immobilised antiglobin antibody coupled to colloidal gold particles which are re-suspendible by a passing liquid front while the second section of the second region is an area of immobilised antiglobin capture antibody. The third region comprises an absorbent pad impregnated with guaiac. Alternatively, the third region may comprise a strip of guaiac impregnated paper which is laminated to the second region. It should be understood that the three regions detailed in the present invention may be positioned sequentially or in some other manner, such as superimposed. For example, the first and second regions may be combined such that the sample is deliverable directly into the second region. The test matrix of the present invention may also comprise additional regions. For example, the present invention envisages the use of chromatographic strips which comprises an absorbant pad located after the third region.

Without limiting the present invention to any one theory or mode of action, according to a preferred aspect of the invention the biological sample which is applied to the first region wicks from the first region to the second region and the detection of globin and haem is then performed as a sequential two step procedure. At the second region, the globin component of any haemoglobin which is present in the sample is bound by the antiglobin antibody coupled to the colloidal gold particles. The passing biological sample front re-suspends these antibodies and both the globin-antiglobin complex and the free anti-globin antibody wick from the first section of the second region to the second section. At the second section the globlin component of any haemoglobin present in the sample becomes bound to the immobilised antiglobin capture antibody while free antiglobin coupled to colloidal gold, the non-globin components of the biological sample and any excess globin which is not bound by the anti-globin antibodies of the second region continued to wick into the third region. At the third region, the haem component of any haemoglobin which has not been captured at the second region reacts with a developer solution to cause the release of oxygen, which oxygen reacts with a chromogen such as guaiac to result in a colour change.

(i) applying a gastrointestinal sample to a first region of a test matrix which test matrix comprises first, second and third regions;

(ii) permitting flowing of said gastrointestinal sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin antibody for a time and under conditions sufficient for a globin antiglobin complex to form and detecting said globin antiglobin complex; and

(iii) permitting flowing of said gastrointestinal sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem.

More preferably, said biological sample is a faecal sample. Even more preferably, said chromogen is guaiac or functional equivalent thereof.

Reference to "functional equivalents" should be understood as a reference to fragments, parts, portions, mutants, homologues, mimetics from natural, synthetic or recombinant sources including fusion proteins which exhibit chromogen activity. Derivatives may be derived from insertion, deletion or substitution of amino acids. Amino acid insertional derivatives include amino and/or carboxylic terminal fusions as well as intrasequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which one residue in the sequence has been removed and a different residue inserted in its place. Additions to amino acid sequences include fusions with other peptides, polypeptides or proteins.

capture antibody impregnated in the second section of the second region of the chromatography strip, the colloidal gold becomes visible as a pink band due to its increasing concentration during trapping of the complex at this point. Alternatively, the antiglobin antibody may be radio-labelled or enzymatically labelled such that upon addition of a substrate a colour change is observed if globin is present. The detection of haem by a chromogen is preferably achieved by the addition of a developer such as peroxide which reacts with haem to produce water and oxygen. The oxygen which is liberated then reacts with the chromogen to produce a colour change. For example, when guaiac reacts with oxygen a blue colour is produced. The chromogen may be incorporated into the test matrix at the third region together with the developer or else the developer may be added as a liquid reagent at a later stage. If the developer is dried into the test matrix with the chromogen, then the paper will turn blue upon the arrival of aqueous haemoglobin. Alternatively, some other type of reporter molecule which detects the reactivity between the haem and the chromogen or functional equivalent thereof may be used.

In a preferred aspect, the present invention is used to diagnose gastrointestinal tract bleeding by analysing faecal samples for the presence of blood. Without limiting the present invention to any one theory or mode of action, the chromogen test will positively identify bleeding from any part of the gastrointestinal tract (that is, both the upper and lower regions of the tract) since it detects the haem component of haemoglobin and haem is relatively resistant to breakdown in the small intestine (the upper gastrointestinal tract). The globin component of haemoglobin however, does not survive passage through the upper gastrointestinal tract. A positive globin result in a faecal sample therefore indicates that bleeding has occurred in the lower gastrointestinal tract. Accordingly, by applying a combined two step immunological and non-immunological based test, it is possible to differentiate between upper and lower gastrointestinal tract bleeding wherein a positive haem result together with a negative globin result indicates upper gastrointestinal tract bleeding and a positive haem result together with a positive globin result indicates lower gastrointestinal tract bleeding. This is of particular importance, for example to the diagnosis of colorectal cancer, the symptoms of which include lower gastrointestinal tract bleeding.

(iii) permitting flowing of said faecal sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem;

wherein a positive haem result and a negative globin result is indicative of upper gastrointestinal tract bleeding.

Preferably said chromogen is guaiac or functional equivalent thereof.

Yet another aspect of the present invention is directed to a method of diagnosing disease conditions, the symptoms of which include bleeding, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix which test matrix comprises first, second and third regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detecting said globin-antiglobin complex; and

(iii) permitting flowing of said biological sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem.

Preferably, the present invention is directed to a method of diagnosing colorectal cancer said method comprising the steps of:

(i) apply a faecal sample to a first region of a test matrix which test matrix comprises first, second and third regions;

## EXAMPLE 1
## CHROMATOGRAPHIC TEST STRIP

Immunochromatographic tests typically use dried immunological reagents on a test strip. Liquid sample applied to the origin of the test strip flows through the various regions so that with a positive result, a coloured line develops in the upper region of the test strip. The reagents and sample then flow into an absorbent matrix at the top of the test strip. This absorbent is most commonly an absorbent paper, such as blotting paper.

It has now been found that this absorbent paper may be impregnated with guaiac, so that addition of developer solution to the absorbent at the conclusion of the immunological test can enable:

(a) Detection of high levels of blood that may have caused inhibition of the immunological test.

(b) Detection of lower gastrointestinal bleeding.

Alternatively, a strip of guaiac impregnated paper may be inserted at the upper region of the test strip, between the immunological detection zone and the absorbent.

Figure 1 depicts a test matrix suitable for use according to the method of the present invention. The origin and the first section of the second region, which is impregnated with labelled antibody (for example, antiglobinantibody coupled to colloidal gold particles), are made of the same material, which material is a conductive paper (Ahlstrom 1281). The capture antibody which is immobilised in the second section of the second region which region is made of Millipore nitrocellulose. Typically, a functional control line is also included in this region. The chromogen may be impregnated directly in the third region (the absorbent sink) or impregnated in a paper bridge between the second and third regions.

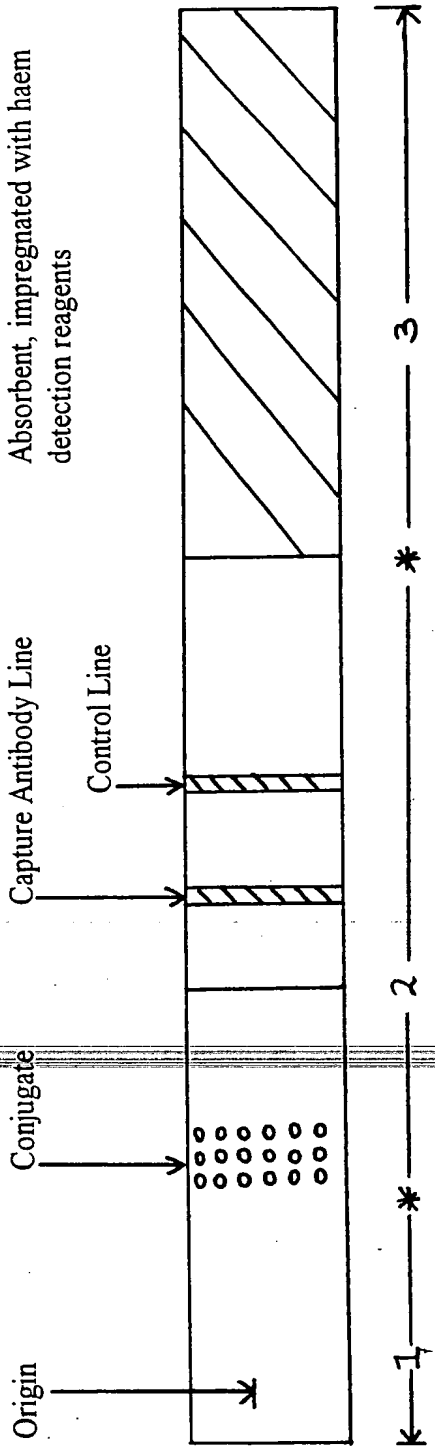
Absorbent, impregnated with haem detection reagents
Capture Antibody Line
Control Line
Conjugate
Origin
1
2
3
Region

Figure 1